Tuberculosis and Other Mycobacteria

Introduction

Tuberculosis remains a major public health problem in the United States, with HIV-infected persons, homeless and under-served populations, and immigrants from endemic areas at particularly high risk for disease. In recent years, tuberculosis control efforts have been complicated by the emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* (MDRTB), some of which are resistant to all first-line antituberculosis drugs. These strains have produced major outbreaks of disease, primarily among HIV-infected persons, in different parts of the country. The increase in the number of tuberculosis cases in the early 1990s, coupled with the emergence of MDRTB, prevented all but 19 states from reaching the Year 2000 goal of 3 cases of tuberculosis per 100,000 population. Re-emphasis of traditional control efforts will be needed to ensure that all states reach this goal in the near future. In addition, new intervention and prevention strategies will be needed to reach the 2010 objective of eliminating endogenous tuberculosis in the United States by the year 2010.

Nontuberculous mycobacterial (NTM) disease also poses an increasing public health challenge. Disseminated NTM infection is a common opportunistic infection in AIDS patients, and increasing numbers of NTM infections are being diagnosed in other immunocompromised as well as nonimmunodeficient populations. Outbreaks due to NTM have also been observed. However, the epidemiologic features and microbiologic aspects of the various components of NTM disease are poorly understood.

The hazards posed by mycobacterial infections emphasize the need for enhanced epidemiologic study, development of new therapeutic regimens, rapid diagnostic procedures, and rapid methods to determine drug susceptibility.

The overall scope of the Tuberculosis/Mycobacteriology Branch, DASTLR, includes studies on the microbiology, molecular biology, and biochemistry of mycobacteria and the pathogenesis and immunology of mycobacterial infections. Activities focus on understanding *M. tuberculosis*, but also include studies involving many other important pathogenic mycobacteria such as *M. avium* and *M. leprae*. Activities include application of gene amplification techniques and DNA probes; detection of unique chemical compounds (e.g., mycolic acids) to improve diagnosis of mycobacterial disease; studies of new drugs for their activity against mycobacteria; identification of genes and gene products for use in new diagnostic procedures or preventive therapies; assistance to state health departments; and support of epidemiologic investigations, including investigations conducted by the Division of Tuberculosis Elimination (DTBE), NCHSTP.

1999 Accomplishments

Laboratory Support

- Provided financial ($10.3 million) and technical assistance to state, city, and territorial public health laboratories to upgrade their mycobacteriology laboratories. Provided

telephone, written, and on-site Epi-aid assistance as requested. Provided telephone consultations on laboratory procedures, laboratory safety, quality control, and quality assurance.

Subtyping

- Funded the six regional laboratories of the National Tuberculosis Genotyping and Surveillance Network, including the Alabama, California, Michigan, New York, and Texas state health department laboratories and the John McClellan Veterans Hospital in Little Rock, Arkansas. Implemented a new quality assurance program for the fingerprint activities.

- Provided restriction fragment length polymorphism (RFLP) support for DTBE/NCHSTP outbreak investigations (Maryland, New York, Tennessee, Georgia). Performed rapid fingerprinting using the mixed-linker PCR method or spoligotyping method to investigate suspected laboratory cross-contamination in Georgia, South Dakota, and Texas. Incorporated the use of polymerase chain reaction (PCR) assays specific for the CDC1551 strain and W strain of *M. tuberculosis* to meet requests from DTBE and referring laboratories.

- Conducted a multi-site evaluation of spoligotyping and VNTR (variable number of tandem repeats) typing and funded Massachusetts and Arkansas to evaluate spoligotyping on a prospective basis for its potential use as a rapid typing tool. Developed a system for designating spoligotypes that provides a unique identifier for all spoligotypes.

- Began development of a PCR/reverse dot blot assay to detect specific sites of insertion of IS6110 as a way to improve the discriminatory power of fingerprinting.

Outbreak Investigation

- Assisted a Florida hospital in investigating of an outbreak of post-operative infections caused by *M. abscessus* by typing isolates using multi-locus enzyme electrophoresis. All 8 patient isolates tested were identified as *M. abscessus* with electrophoretic types ET1 (5 isolates) or ET2 (3 isolates). One environmental isolate was *M. abscessus* but had a different electrophoretic type. Ten other environmental isolates were identified as *M. mucogenicum.*

- Supported the Hospital Infections Program (HIP), NCID, in the investigation of an outbreak of *M. mucogenicum* associated with central intravenous catheters in a Minneapolis medical center. None of the patient isolates matched by electrophoretic type, but one patient isolate had the same electrophoretic type as three environmental water isolates.

- Supported an investigation by HIP/NCID and the California Department of Health involving *M. fortuitum* wound infections that occurred in three patients after implantation

of infusion pumps. The isolates from the patients had matching electrophoretic types.

- Assisted an investigation of a cluster of five unusual *M. avium* isolates detected in an Oregon laboratory. The isolates displayed the same electrophoretic type. Hospital officials concluded the isolates originated from cross-contamination and did not represent true infections.

- Investigated a cluster of *M. chelonae* and *M. mucogenicum* isolates from a California laboratory. Seven isolates were identified as *M. chelonae* and shared the same electrophoretic type, posssibly representing cross-contamination. Two of the six *M. mucogenicum* isolates had an identical enzyme profile, while the remaining four isolates appeared to have unique profiles.

- Identified and typed isolates from bronchoscopy specimens from ten patients and one tap water isolate submitted by an Illinois Hospital laboratory as *M. avium* complex. One patient isolate and the water isolate were *M. avium* and shared the same electrophoretic type. Nine patient isolates were *M. intracellulare* and displayed four electrophoretic types, with five isolates sharing the same electrophoretic type.

Reference Testing

- Maintained an in-house quality assurance program to enable the reference laboratory to provide timely results to referring laboratories and to assure compliance with Clinical Laboratories Improvement Act (CLIA) regulations.

- Identified 636 referred isolates, predominantly NTM, for state and other authorized health facilities using high-performance liquid chromatography (HPLC) analysis of mycolic acids. Provided telephone consultations/interpretations of HPLC patterns. Updated the collection of HPLC patterns of mycolic acids with those of 14 recently described/named species of *Mycobacterium*, bringing the total number of isolate patterns in our library to 81. Prepared Mycolic Acid Patterns Standards monograph for electronic distribution via the website.

- Identified and characterized two new species of *Mycobacterium* - *M. kubicae* and *M. septicum.*

- Implemented routine use of PCR-RFLP and allele-specific PCR for differentiating *M. tuberculosis* from *M. bovis* based on polymorphism in *oxyR.*

- Implemented quadruplex PCR-RFLP analyses of insertion elements (IS1245 and IS6110) and polymorphic genes (65-Kda HSP and 16S rRNA) as routine adjunct tests for identifying pathogenic *Mycobacterium* species.

- Performed agar proportion drug susceptibility tests on 460 slow-growing mycobacterial

isolates, predominantly *M. tuberculosis*, submitted by state and other authorized health facilities for patient care. Performed 180 minimum inhibitory concentration (MIC) determinations on rapidly growing mycobacteria. Reincorporated the use of the BACTEC TB system for susceptibility testing of pyrazinamide (PZA).

- Provided susceptibility testing of 70 *M. tuberculosis* isolates and RFLP analysis and DNA sequence analyses on rifampin-resistant strains *(rpoB*) in support of the DTBE/NCHSTP rifapentine clinical trial as well as susceptibility testing of 33 isolates in support of three new DTBE clinical trials.

Resistance

- Optimized and standardized laboratory procedures for DNA sequence analyses of *M. tuberculosis* drug resistance markers and began incorporation of genetic tests for susceptibility testing *(rpoB, pncA)* into the routine workflow.

- In collaboration with DTBE/NCHSTP and the California Department of Health, characterized PZA mono-resistant strains of *M. tuberculosis* using DNA sequence analyses of the *pncA* gene and the *oxyR* gene.

- Collaborated with the Division of Bacterial and Mycotic Diseases (DBMD), NCID, to study clarithromycin resistance among sterile-site isolates of *M. avium* complex (MAC) among persons in Atlanta and Houston. Isolates from 8 of 54 patients from Atlanta and 6 of 16 patients from Houston were resistant to clarithromycin. The patients with drug-resistant isolates were seven times more likely than patients with drug-susceptible isolates to have received macrolide therapy within the 2-month period prior to the development of disease.

- Analyzed mutations in the pyrazinamidase gene (*pncA*) in a set of 60 *M. tuberculosis* clinical isolates. Identified 30 mutations, 17 of which were previously unknown, and demonstrated the association of genetic mutations with phenotypic levels of PZA resistance determined by BACTEC and agar proportion methods.

- Sequenced portions of the *embB* gene among 40 ethambutol resistant *M. tuberculosis* isolates and developed a rapid single-strand conformation polymorphism screening method to identify *embB* mutations.

- Analyzed occurrence of specific *rpoB* mutations in spontaneous *in vitro*-selected, rifampin-resistant *M. tuberculosis* strain H37Rv. Determined that the most common mutations seen in clinical isolates, especially serine 531 to leucine, also occur at high frequency in culture.

- Compared *M. tuberculosis* strains H37Rv and CDC1551 and three members of the extended W family and found that they all had similar rates of mutation to resistance for

isoniazid, rifampin, streptomycin, and ciprofloxacin.

- Examined 10 isolates of *M. tuberculosis* that contained a population of resistant and susceptible bacteria (i.e., <100% resistance to rifampin in the Methods of Proportion assay). For 6 isolates, only one mutation was detected among the rifampin-resistant colonies. For 4 isolates, two or more different mutations were found among the rifampin-resistant colonies. The presence of multiple mutants in a clinical sample may complicate detection of resistance by genotypic assays.

- Evaluated a linear signal amplification ("Invader") method for the rapid identification of drug-resistance associated mutations. Determined that mutations present at a frequency $\geq$0.5% of the population could be detected by this method.

- Began evaluation of techniques to identify mutations associated with drug resistance by temperature-mediated heteroduplex analysis using a nucleic acid HPLC instrument and by measuring melting curves of fluorescent duplex DNA molecules.

- Distributed a coded challenge set of *M. tuberculosis* strains with known drug-susceptibility patterns to manufacturers conducting trials of new susceptibility testing assays in collaboration with the Food and Drug Administration (FDA).

Pathogenesis

- Developed a membrane based miniarray to monitor expression of *M. tuberculosis* genes among various strains and growth conditions using psoralen labeled probes. This assay allowed for the evaluation of RNA preparation and labeling techniques to be used in microarray assays.

- Identified a monoamine oxidase (MAO) homolog in *M. tuberculosis* and produced a polyclonal antibody to it. The MAO gene was cloned into a mycobacterial expression vector and put into *M. smegmatis* to facilitate the search for its substrate. MAO may play a role in isoniazid resistance.

- Demonstrated induction of both HIV/BaL (R5 tropic) and HIV/Lai (X4 tropic) production during co-infection of peripheral blood mononuclear cells (PBMCs) with *M. tuberculosis*, but not *M. bovis* strain BCG. Both T-cells and macrophages are involved in the amplified viral replication. Levels of T-cell activation markers, CD25 and CD69, and cytokines, TNF-alpha, IFN-gamma, RANTES and IL-8, were increased in co-infected cells.

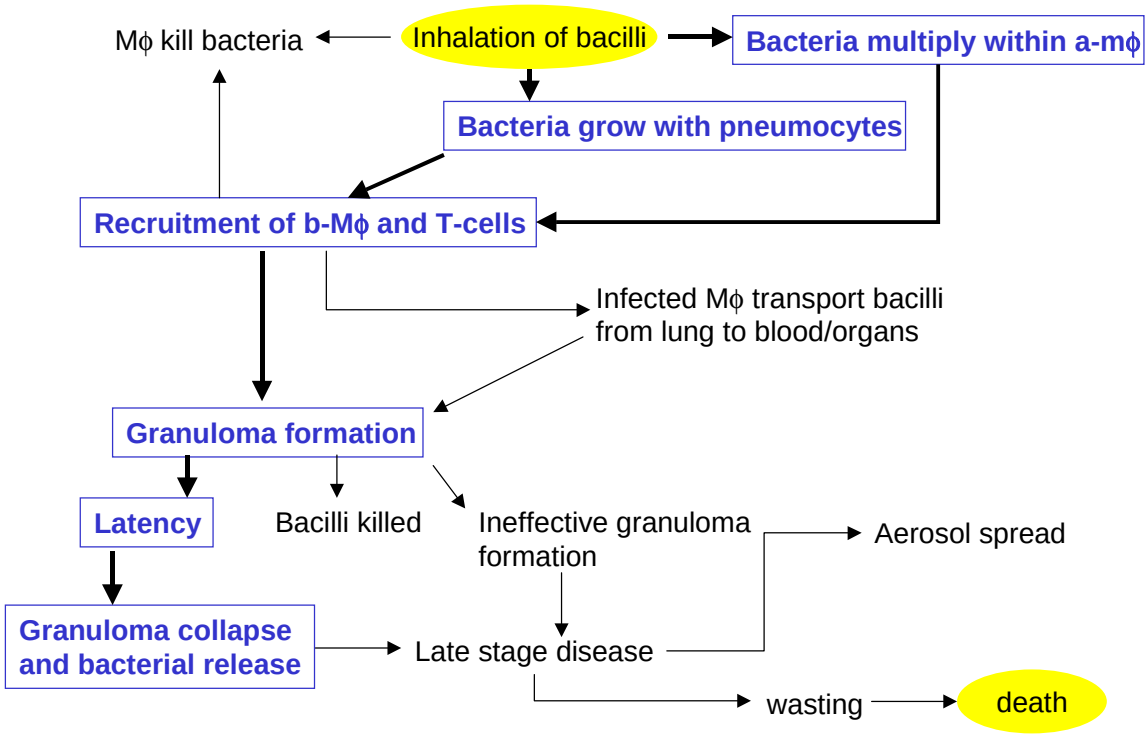
- Observed *M. tuberculosis* bacilli attaching to actin fibrils protruding from the A549 cell surface. Identified two *M. tuberculosis* antigens that possess affinity for lung epithelial cell (A549) membranes. One antigen is the alpha-crystallin protein (Acr). Began producing monoclonal antibodies against these antigens for use in the characterization of their roles in attachment.

- Demonstrated that the addition of either live *M. tuberculosis* bacilli or bacterial lysates to the apical surface of the bilayer model induced the production of both chemokines and cytokines, resulting in the migration of lymphocytes and mononuclear cells from the basal to the apical sides. On the apical surface, granuloma formation was observed to occur in close association with epithelial cells.

- Determined that viable intracellular mycobacteria in three-week-old *in vitro* granulomas had thickened cell walls similar to bacilli incubated for a similar length of time in the anoxic chamber. Increased levels of IL-1-beta, IL-4, IL-6, IL-8, IL-12, TNF-alpha, IFN-gamma, RANTES and ICAM-1 were produced by the cells in the granuloma. The addition of exogenous TNF-alpha and IL-8 enhanced the rate of granuloma formation.

- Developed a quantitative RT-PCR assay to examine *M. tuberculosis* gene expression during growth and survival in the anoxic chamber latency model, *in vitro* granuloma model, and guinea pig models. Sets of primers and probes were designed to identify all 13 *M. tuberculosis* sigma factor cDNAs.

Working model of *M. tuberculosis* pathogenesis



- Observed differences in virulence between human clinical and non-clinical strains of *M. avium*. All five clinical strains grew significantly faster and were more toxic to macrophages compared to the non-clinical strains.

- Identified a potential environmental reservoir for *M. marinum*. Two strains of *M. marinum* cultured in *Acanthamoeba polyphaga* were found to be significantly more cytopathic to SW872 human adipose cell monolayers than the same strains prior to passage.

- Using an agarose encapsulation assay, showed that partially purified, secreted toxin(s) from *M. ulcerans* produced necrosis prior to apoptosis on human adipose cells.

Training and Evaluation Activities

- Participated in a multi-laboratory consortium, developed in collaboration with PHPPO, to assess method-dependent performance problems with *M. tuberculosis* drug-susceptibility testing including inter- and intra-laboratory reproducibility of results from testing first-line drugs, the frequency of low-level INH resistance, and the frequency of ethambutol mono-resistance.

- Provided expertise for PHPPO's *M. tuberculosis* Nucleic Acid Amplification Testing Performance Evaluation Program and the Mycobacterial Drug Susceptibility Testing Performance Evaluation Program.

- In collaboration with PHPPO, conducted a mycobacteriology laboratory training needs assessment, and began to update the laboratory manual "Public Health Mycobacteriology: a Guide for the Level III Laboratory."

- In collaboration with DTBE/NCHSTP, developed guidelines for the laboratory diagnosis of *M. tuberculosis* infections by nucleic acid amplification techniques, and provided technical assistance and expertise for contracts supporting "Assessing the Clinical and Economic Impact of Nucleic Acid Amplification Assays on Tuberculosis."

- Participated in the American Thoracic Society/CDC working group on guidelines for the use and interpretation of drug-susceptibility tests for *M. tuberculosis*, and the National Committee for Clinical Laboratory Standards (NCCLS) working group on performance of mycobacterial drug susceptibility testing.

Plans for 2000

Laboratory Support

- Provide technical and financial support for upgrading state, city, and territorial mycobacteriology laboratories. Conduct site visits of public health mycobacteriology

laboratories to assess program accomplishments and identify critical unmet needs.

Subtyping

- Support and oversee regional laboratories of the National Tuberculosis Genotyping and Surveillance Network. Complete enrollment of patients for the Sentinel Surveillance Project and analyze Network data. Conduct annual meeting of the Network in Atlanta and formulate plans for activities of the Network following completion of the Sentinel Surveillance Project.

- Assist regional labs in implementing spoligotyping and submitting spoligotype results to the national database. Determine feasibility of preparing and distributing spoligotyping membranes and reagents for use in the Genotyping Network.

- Provide RFLP support for CDC outbreak investigations and international studies. Perform rapid fingerprinting when needed. Evaluate the use of spoligotyping as a routine secondary typing method. Perform DNA sequence analysis and other molecular genetic analysis as needed in support of epidemiologic investigations.

- Develop and evaluate a PCR/reverse dot blot assay for detecting insertion sites of IS6110 and typing strains. Refine assay by including additional insertion sites.

Reference Testing

- Develop and evaluate a method for typing *M. bovis* and *M. tuberculosis* based on the polymorphic GC-rich repetitive sequences (PGRS).

- Provide laboratory support to CDC, state health departments, and medical facilities investigating outbreaks and cross-contamination due to nontuberculous mycobacteria.

- Develop PCR-based assays to detect DNA polymorphisms corresponding to variations detected by multilocus enzyme electrophoresis for rapid fingerprinting of nontuberculous mycobacteria.

- Provide identification and drug-susceptibility testing for isolates referred from state and other authorized health facilities and in support of DTBE/NCHSTP investigations. Reincorporate the use of conventional biochemical tests and nucleic acid probes into the identification workflow. Implement the routine use of BACTEC for PZA susceptibility testing and develop BACTEC for testing second-line agents.

- Provide laboratory support for three clinical trials evaluating new therapies for tuberculosis conducted by DTBE/NCHSTP.

- Provide laboratory support for a DBMD/NCID surveillance project to detect

clarithromycin resistance among sterile-site isolates of *M. avium* complex.

- Perform taxonomic analysis of unidentified mycobacteria using HPLC, sequencing of the 16S rRNA gene, and PCR-RFLP analysis of 65-kDa antigen gene to identify new species. Update the collection of HPLC patterns of mycolic acids with those of recently described/named species of mycobacteria.

- Incorporate the identification of *M. chelonae* and *M. abscessus* isolates as a routine procedure. Determine if HPLC patterns can be used to reliably distinguish these two species.

Resistance

- Evaluate the ability of Invader technology and temperature-mediated heteroduplex analysis by nucleic acid-HPLC to detect mutations associated with drug resistance.

- Characterize specific mutations in the *pncA* gene by cloning and expressing the gene in a strain of *M. tuberculosis* in which the native copy of *pncA* is inactivated. Correlate specific mutations with MIC values for PZA.

- Investigate pyrazinamide (PZA) -resistance mechanisms in strains of *M. tuberculosis* that have no detectable *pncA* mutations and fail to synthesize the *pncA* gene product (pyrazinamidase).

- Distribute a coded challenge set of *M. tuberculosis* strains with known drug-susceptibility patterns to manufacturers conducting trials of new susceptibility testing assays, participate in analysis of results of testing in collaboration with the FDA, and provide assistance to manufacturers in analysis of the results.

Pathogenesis

- Characterize the effect of resuscitation factor to the recovery and growth of *Mycobacterium paratuberculosis.* Initiate studies to assess drug susceptibilities of M. paratuberculosis isolates.

- Apply techniques developed with membrane based miniarrays to microarrays to monitor expression of *M. tuberculosis* genes among various strains and growth conditions to identify virulence factors of *M. tuberculosis* and potential diagnostic, vaccine or therapeutic targets.

- Characterize gene expression, in vitro and in vivo growth characteristics, and ability to induce an immune response in well-documented *M. tuberculosis* strains with low and high rates of transmission.

- Characterize the expression of MAO and details of its the enzymatic activity and substrate specificity in *M. tuberculosis.*

- Examine the potential use of Acr as a diagnostic of *M. tuberculosis* latency by testing sera from individuals with active disease or latent *M. tuberculosis* infections for the presence of Acr or antibodies to Acr

- Evaluate the role of Acr in the attachment of tubercle bacilli to pneumocytes using recombinant Acr protein, anti-Acr antibodies and a strain of *M. tuberculosis* lacking the *acr* gene. Evaluate the role of the second putative *M. tuberculosis* epithelial cell adhesin using monoclonal antibodies.

- Screen *M. tuberculosis* cell extracts, as well as *M. smegmatis* and *M. bovis* BCG libraries containing *M. tuberculosis* chromosomal fragments, to identify the bacterial component(s) responsible for the immune induction and viral amplification observed during HIV/*M. tuberculosis* co-infection. Characterize identified components in an *ex vivo* cell culture model.

- Measure cytokine and cell surface molecule expression in the lung bilayer model at time points later than 48-hours post-infection. Monitor cell trafficking using fluorescent host cells and analyze affect of blocking antibodies on lymphocyte and macrophage migration, and granuloma formation and maintenance.

- Examine granulomas recovered from lungs of *M. tuberculosis*-infected guinea pigs for the induction of host genes encoding cytokines and surface markers. Use microprobe technology in collaboration with the Center for Biofilm Engineering at Montana State University to measure the internal structure, pH and oxygen content of *in vitro* and animal granulomas.

- Characterize the regulation of sigma factor-E expression and determine whether SigE expression is essential for pathogenesis using a *sigE* knock-out mutant of *M. tuberculosis*. Identify which genes are being regulated by SigE comparing two-dimensional gel electrophoresis of total cell proteins in strains constitutively expressing *sigE* versus with that of the *sigE* knock-out mutant. Similar studies will be conducted with the 12 other known sigma factors of *M. tuberculosis*.

- Use a skin raft model incorporating adipose cells to study the migration of live *M. ulcerans* bacteria through the various skin layers toward the adipose cells. Characterize the pathology and immune response against various *M. ulcerans* antigens, including the toxin(s), in a guinea pig model. Initiate antitoxin and toxoid efficacy studies.

Training and Evaluation Activities

- Assess the specificity and sensitivity of an optical sensor diagnostic system for

mycobacteria through a cooperative research and development agreement with Photonic Sensor Systems, Inc.,

- Complete update of the laboratory manual "Public Health Mycobacteriology: a Guide for the Level III Laboratory" in collaboration with PHPPO.

- Provide technical assistance and expertise for DTBE/NCHSTP contracts supporting "Assessing the Clinical and Economic Impact of Nucleic Acid Amplification Assays on Tuberculosis."

- Participate in the American Thoracic Society/CDC working group on guidelines for the use and interpretation of drug susceptibility for *M. tuberculosis*, and the National Committee for Clinical Laboratory Standards working group on performance of mycobacterial drug susceptibility testing.

- Provide expertise for PHPPO's *M. tuberculosis* Nucleic Acid Amplification Testing Performance Evaluation Program and the Mycobacterial Drug Susceptibility Testing Performance Evaluation Program.

- Participate in PHPPO's multi-laboratory consortium to assess method-dependant performance problems with *M. tuberculosis* drug-susceptibility testing.